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Cloning and Nucleotide Sequence of the *Campylobacter jejuni gyrA* Gene and Characterization of Quinolone Resistance Mutations

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The *gyrA* gene of *Campylobacter jejuni* UA580, which encodes the A subunit of DNA gyrase, was cloned and its nucleotide sequence was determined. An open reading frame of 2,589 nucleotides was identified, which could code for a polypeptide of 863 amino acids with a M_r of 97 kDa. Both the nucleotide sequence and the putative amino acid sequence show ca. 50% identity with those of other *gyrA* genes from gram-positive and gram-negative bacteria. The locations of the *gyrA* gene on genome maps of both *C. jejuni* UA580 and *Campylobacter coli* UA417 were determined. Six nalidixic acid-resistant isolates of *C. jejuni* were shown to carry mutations in *gyrA*. Three clinical isolates had Thr-86-to-Ile substitutions. Three laboratory mutants had substitutions of Thr-86 to Ile, Asp-90 to Ala, and Ala-70 to Thr, respectively. The mutation at Thr-86, which is homologous to Ser-83 in *Escherichia coli*, was associated with high-level resistance to ciprofloxacin in *C. jejuni*.

DNA gyrase, a type II DNA topoisomerase, is essential for bacterial viability. It catalyzes ATP-dependent negative supercoiling of DNA and is involved in DNA replication, recombination, and transcription (33). The enzyme consists of two A and two B subunits, which are encoded by the genes *gyrA* and *gyrB*, respectively. The nucleotide sequences of several *gyrA* genes have been reported, including those of *Bacillus subtilis* (20), *Escherichia coli* (29), *Klebsiella pneumoniae* (4), and *Staphylococcus aureus* (18). In *B. subtilis* and *S. aureus*, gyrase genes are organized in the order *gyrB-gyrA* and are located close to the origin of replication (18, 20). In *E. coli* and *K. pneumoniae*, the *gyrA* and *gyrB* genes are located separately on the chromosomes (4, 12).

DNA gyrase inhibitors such as nalidixic acid and the quinolone ciprofloxacin exert their potent antagonistic effects on bacterial growth by interfering with the GyrA protein (6, 28). These drugs have been widely used to treat bacterial infections, including those due to *Campylobacter jejuni* and *Campylobacter coli* (1, 8, 26), which are a major cause of bacterial diarrhea in humans. High-level resistance to quinolones has been reported in *Campylobacter* spp. both in vitro (32) and in patients treated with fluoroquinolones (1, 26). In *E. coli*, mutations conferring quinolone resistance were found in both genes of DNA gyrase; however, mutations responsible for high-level resistance were mapped mainly to the *gyrA* gene (12). Quinolone resistance mutations in the GyrA protein have been identified from the DNA sequence analysis of quinolone-resistant *gyrA* genes, including Ser-83 → Leu, Ser-83 → Trp, Asp-87 → Asn, Gly-81 → Cys, Ala-84 → Pro, Ala-67 → Ser, and Gln-106 → His (39). Mutations in Ser-83, in particular, have been found in the majority of quinolone-resistant *E. coli* clinical isolates (21). Similar mutations were also identified in ciprofloxacin-resistant isolates of *S. aureus* (10, 27).

Gootz and Martin (9) demonstrated that the DNA gyrases from Nal^r mutants of *C. jejuni* UA535 were 100-fold less susceptible than the wild-type enzyme to inhibition by quinolones in the DNA supercoiling reaction. Subunit switching experiments with purified A and B subunits from the wild type and one of the quinolone-resistant mutants indicated that an alteration in the A subunit was responsible for resistance. Here, we report the cloning and nucleotide sequence of the *C. jejuni gyrA* gene and the location of the gene on both *C. jejuni* and *C. coli* chromosomes. Several mutations responsible for quinolone resistance were detected in the *gyrA* sequence.

MATERIALS AND METHODS

Strains and culture conditions. The *Campylobacter* spp. employed in this study were *C. jejuni* UA67 (Nal^r mutant [35]); UA536, UA543, and UA549 (Nal^r clinical isolates from H. Lior); UA580 (35); UA580R1 and UA580R3 (Nal^r mutant from UA580 [this study]); and *C. coli* UA417 (Nal^r clinical isolates [35]). *E. coli* DH5α (23) was also used. The plasmids and phages employed were pUC19, M13mp18, M13mp19 (38), pBluescript II SK (Stratagene), pK194 (16), and pT7-5 (30).

Campylobacters were grown at 37°C on Mueller-Hinton agar medium containing 7% CO_2. *E. coli* was grown in 2× YT medium or on Luria-Bertani agar (23) at 37°C. When necessary, the medium was supplemented with ampicillin (100 μg/ml), kanamycin (15 μg/ml), or nalidixic acid (24 μg/ml).

DNA isolation, transformation, and nucleotide sequence analysis. Plasmid DNA was isolated by a modification of the alkaline lysis method of Birnboim and Doly (2) and purified by the "magic miniprep" (Promega) when used for restriction analysis and sequencing. M13 phage DNA was prepared by the method described by Sambrook et al. (23). Chromo-

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somal DNA was isolated by sodium dodecyl sulfate (SDS) lysis, followed by phenol-chloroform extraction. *E. coli* was transformed by the $CaCl_2$ procedure (23). Single-stranded M13 DNA and asymmetric polymerase chain reaction (PCR) products were sequenced by the dideoxy chain termination method by using Sequenase (United States Biochemical Corp.) following the manufacturer's instructions.

Nucleotide and derived amino acid sequences were analyzed by the Inteligenetics Sequence Analysis Program or the Multiple Alignment Construction and Analysis Workbench (25).

PCR. Two degenerate primers for the synthesis of the *gyrA* probe were derived from codons 39 to 45 and 173 to 179 of the *E. coli gyrA* gene (15). Two primers for detecting the *gyrA* gene mutations are indicated in Fig. 2.

PCR amplification was performed in 100-μl reaction mixtures containing 1× buffer (Promega), 1.5 mM $MgCl_2$, 100 μM dCTP and dGTP, 200 μM dATP and dTTP, 50 pmol of each primer, 400 ng of genomic DNA, and 2.5 U of *Taq* DNA polymerase (Promega). The reaction was carried out for 30 cycles in which the conditions were 50 s at 90°C for denaturation (the midpoint temperature for campylobacter DNA is estimated to be 83°C), 50 s at 52°C for annealing, and 30 s at 72°C for extension.

Asymmetric PCR was performed with 5 μl of unpurified PCR product as the template. The reaction was carried out under the same conditions except that only one primer was added. PCR products were electrophoresed on 1.2% agarose gels to check for the presence of single-stranded DNA and then were purified by spin dialysis (23) or from low-melting-point agarose gels.

DNA hybridization and PFGE. DNA was labeled by the random-primer labeling method with [^{32}P]dCTP (5). Southern transfer was performed with pure nitrocellulose membranes by the standard method (23). Colony hybridization was carried out with nitrocellulose discs (23) or Whatman filter paper 54 by a modified method of Gergen et al. (7).

Pulsed-field gel electrophoresis (PFGE) was performed as described previously (31).

Nucleotide sequence accession number. The GenBank accession number for the *C. jejuni gyrA* gene is L04566.

RESULTS

Cloning the *C. jejuni gyrA* gene. In spite of the similarities in amino acid sequence identified among the known type II DNA topoisomerase genes (14), no *Campylobacter gyrA* gene was identified when heterologous *gyrA* gene clones were used as hybridization probes (34). Therefore, two degenerate primers from two conserved regions in the N-terminal portion of bacterial *gyrA* proteins (15) were designed and were used to amplify *C. jejuni* chromosomal DNA in a PCR reaction. The PCR product had the expected size of 423 bp, and its sequence was determined after the fragment was cloned into a pBluescript II SK vector (Stratagene). This sequence extends from nucleotide numbers 409 to 832 in the complete sequence of the *gyrA* gene shown in Fig. 2. This cloned fragment was used as the hybridization probe, and it was found to hybridize with a 5-kb *Bgl*II fragment of *C. jejuni* UA580 chromosomal DNA and an 8-kb *Bgl*II fragment of *C. coli* UA585 DNA.

UA580 DNA (0.8 μg) was cleaved with *Bgl*II, ligated to the *Bam*HI-cut pUC19 (0.2 μg), and transformed into *E. coli* DH5α. Nine hundred white, ampicillin-resistant colonies were selected and replicated onto nitrocellulose membranes. The membranes were hybridized with the ^{32}P-labeled PCR product. Six colonies were selected which contained sequences homologous to the probe as judged by strong hybridization signals. Plasmid DNA was isolated from these six recombinants, and four of them contained a 5-kb insert fragment. One of these recombinant plasmids (named pYW75) was mapped with various restriction enzymes as shown in Fig. 1. Southern hybridization showed that the *gyrA* probe hybridized to the 1.2-kb *Eco*RV fragment and the 2.0-kb *Eco*RI-*Eco*RV fragment of pYW75. Preliminary nucleotide sequencing of these two DNA fragments showed that they were highly homologous to the nucleotide sequences of *gyrA* genes from *B. subtilis* and *S. aureus*. The data also indicated that pYW75 did not contain the 3′ end of the *gyrA* open reading frame (ORF).

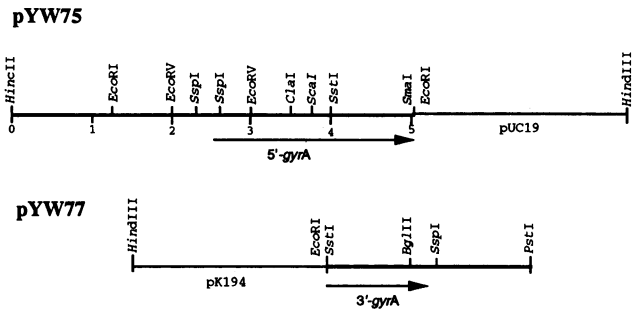

FIG. 1. Restriction maps of cloned *C. jejuni* DNA fragments of pYW75 and pYW77. Thin lines represent the vectors pUC19 and pK194. There is an additional *Sst*I site in pUC19 at 5.2 kb. Arrows indicate the *gyrA* ORF. Numbers are kilobases.

In order to clone the 3′ end of the *gyrA* gene, the 1.2-kb *Sst*I fragment from pYW75, which contains the central portion of the *gyrA* gene, was used as a probe in a Southern hybridization. *C. jejuni* UA580 chromosomal DNA digested with restriction enzymes *Sst*I, *Sst*I-*Eco*RV, *Sst*I-*Hin*dIII, *Sst*I-*Pst*I, and *Sst*I-*Ssp*I was used to prepare the Southern blot. The probe was found to hybridize with a 1.5-kb *Sst*I-*Ssp*I fragment of *C. jejuni* UA580 DNA. Therefore, UA580 DNA was digested with *Sst*I and *Ssp*I, inserted into *Sst*I- and *Hin*cII-cut pUC19 and pK194 (a pACYC184 derivative [16]), respectively, and introduced into *E. coli* DH5α. Two hundred pUC19 recombinants and 50 pK194 recombinants were selected. Colony blots showed that only one pK194 derivative hybridized with the 1.2-kb *Sst*I fragment. The restriction map of this plasmid (named pYW77) is shown in Fig. 1. It proved impossible to subclone either the 1.5-kb *Sst*I-*Ssp*I fragment or the whole 2.4-kb fragment from pYW77 into pUC19, leading us to conclude that cloning and/or expression of the *C. jejuni* gyrase A C terminus may be lethal to *E. coli* host cells. Construction of a complete *gyrA* gene by splicing portions of both pYW75 and pYW77 plasmids into pUC19 or pT7-5 (a pBR322 derivative [30]) was also unsuccessful.

Nucleotide sequence of the cloned UA580 DNA and comparisons of *gyrA* genes. The restriction fragments which hybridized with the *gyrA* probe were subcloned into M13mp18 and M13mp19 phages. Serial deletions were generated by digestion of pYW75 with appropriate restriction enzymes and exonuclease III and subcloned into M13 phage. The whole 5.1-kb fragment of pYW75, however, could not be cloned into the M13 phage. The 1.5-kb *Sst*I-*Ssp*I fragment from pYW77 was also subcloned into M13 phage. Sequencing of these subclones revealed a potential Shine-Dalgarno sequence followed by an ATG start codon and an ORF of 2,589

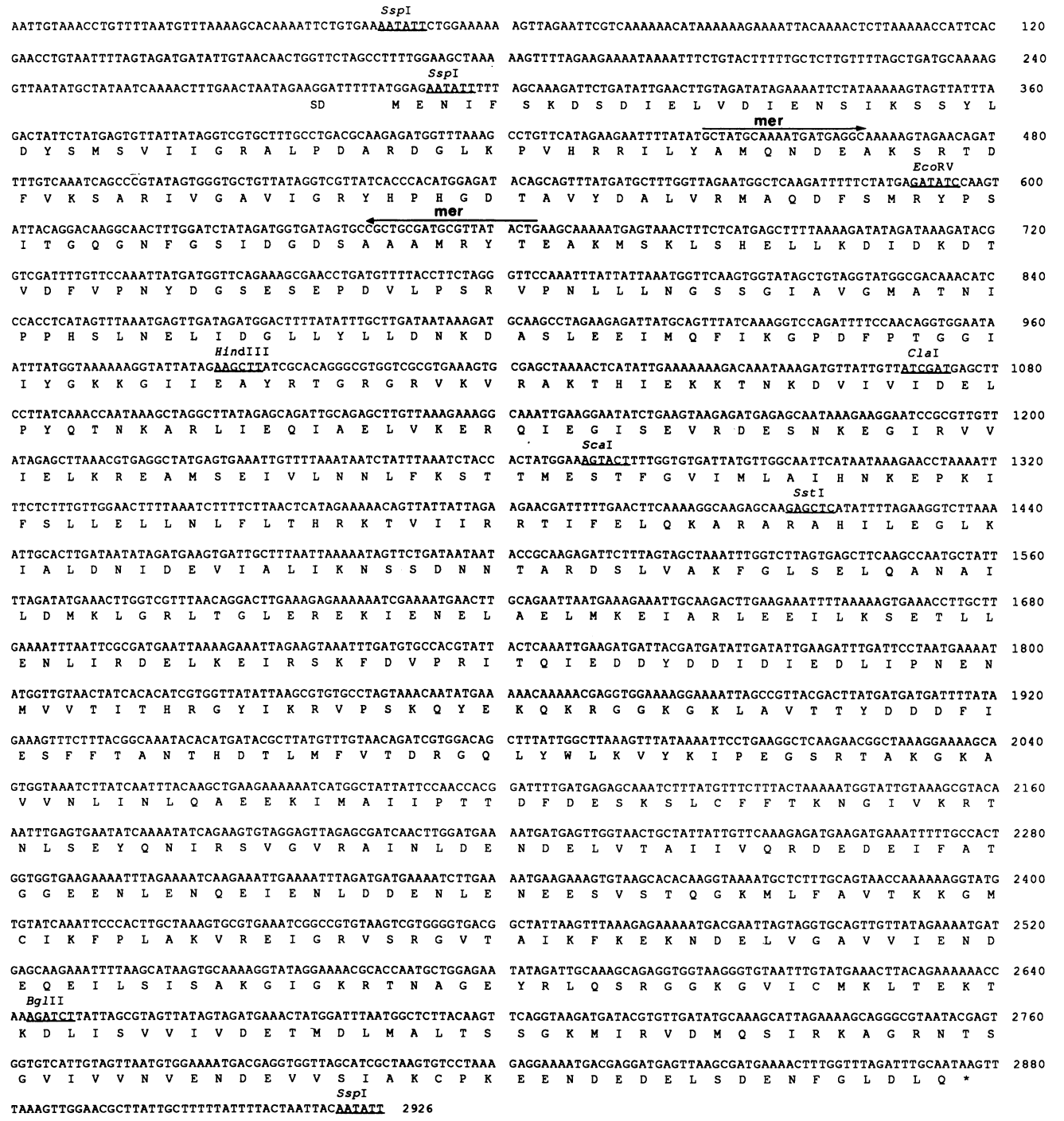

FIG. 2. Nucleotide sequence of the DNA fragment containing the *C. jejuni gyrA* gene. The deduced amino acid sequence of the GyrA protein is also presented. Two sequences of two primers for the PCR amplification are indicated as mer. SD, Shine-Dalgarno sequence.

bp (Fig. 2). This ORF could code for a polypeptide of 863 amino acids with a calculated M_r of 96,985, which is consistent with the gyrase A protein molecular mass of 95 kDa estimated by Gootz and Martin (9). Upstream from the Shine-Dalgarno sequence, there were several sequences similar to the −10 *E. coli* promoter consensus sequence TATAAT; however, no apparent −35 region (TTGACA) was identified. The presence of a −10 sequence without a −35 region was found in both the *E. coli* (29) and *K. pneumoniae* (4) *gyrA* promoters.

From the other direction, there was another possible ORF in the 3′ end of the *gyrA* gene, which could code for a polypeptide of 167 amino acids. However, no corresponding protein was detected (data not shown) in the *E. coli* "maxicell" labeling system (24) or the T7 RNA polymerase expression system (30).

The nucleotide sequence in the *C. jejuni* UA580 *gyrA* coding region exhibits about 50% identity with all other known *gyrA* sequences. The derived amino acid sequence of the *C. jejuni* GyrA protein when compared with other GyrA sequences (Fig. 3) showed 51% overall identity with the *B. subtilis* GyrA, 49% with the *E. coli* and *K. pneumoniae* GyrA, and 48% with *S. aureus* GyrA. As expected, a higher degree of sequence similarity was found near the N-terminal region of the protein. The multigene alignment (Fig. 3) suggests that the Tyr-125 residue of *C. jejuni* is equivalent to the Tyr-122 of *E. coli* with which a transient covalent DNA-protein bridge forms during the double-strand passage reaction of DNA topoisomerization (13). The C-terminal region of the GyrA protein is generally more variable, as consensus amino acids are found with less regularity (22). The GyrA protein alignment further shows that near the amino acid block 710 (Fig. 3), a region of 35 amino acids is uniquely present in the *C. jejuni* UA580 sequence. It would be of interest to determine whether these amino acids are present in other *Campylobacter* species.

In *E. coli*, a gene essential for chromosome segregation, the *parC* gene, is found to be homologous to the *gyrA* gene, which is a subunit of topoisomerase IV (17). The *C. jejuni* GyrA protein reported here shares 35% overall amino acid homology with that of the *E. coli* ParC protein. Although the similarity is higher near the N-terminal ends of the proteins, the C-terminal ends are dissimilar, with few clusters of identical amino acids. Although the identification of the *C. jejuni gyrA* gene product is based on sequence alignment, the overall similarity with the *E. coli* and other GyrA proteins makes it unlikely that the gene described here is the *parC* gene homolog of *C. jejuni*.

Location of the *gyrA* gene. The position of the *gyrA* gene was determined on the genomic maps of both *C. jejuni* UA580 and *C. coli* UA417 (31) by using PFGE and Southern hybridization with the *gyrA* gene probe (Fig. 4). The results showed that in both *Campylobacter* species, *gyrA* is located a considerable distance from the *gyrB* gene. This information is consistent with accumulated data suggesting that the physical separation between the *gyrB* and *gyrA* genes can be correlated with phylogenetic grouping on the basis of 16S rRNA sequences (36). It appears that the two gyrase genes are widely separated in purple bacteria (36). Examples of this type are found in *E. coli* and *Salmonella*, *Klebsiella*, *Pseudomonas*, and *Campylobacter* spp., as demonstrated in this study. Among the nonpurple bacteria, such as *Bacillus*, *Mycoplasma*, and *Staphylococcus* spp., the gyrase genes are located adjacent to one another.

Detection of *gyrA* gene mutations associated with 4-quinolone resistance. Amino acid changes within a small region at the N-terminal end of the GyrA protein have been shown to confer 4-quinolone resistance in *E. coli* and *Staphylococcus* spp. (10, 27, 39). Spontaneous Nal^r mutants were isolated from UA580 by plating fresh UA580 cells onto Mueller-Hinton agar containing nalidixic acid (24 μg/ml). The mutation frequency for Nal^r in this strain was about 5×10^{-7}, which is 10-fold higher than those for several other strains tested (32, 35).

Two primers were synthesized (indicated in Fig. 2) and used to amplify *C. jejuni* and *C. coli* chromosomal DNA isolated from Nal^r laboratory mutants and clinical isolates. A single DNA fragment of ca. 250 bp was obtained from each *C. jejuni* DNA used, but no PCR products were detected with two *C. coli* DNAs as templates. Lowering the annealing temperature to 47°C resulted in the production of multiple bands.

The PCR products from *C. jejuni* DNAs were used as templates for a second asymmetric amplification without purification. The nucleotide sequences of asymmetric PCR products were determined. Figure 5 shows that all six quinolone resistance mutants sequenced contain an amino acid change in this region. Mutations at Ala-70, Thr-86, and Asp-90 all resulted in nalidixic acid resistance, and these mutants showed cross-resistance to ciprofloxacin. However, high-level resistance to ciprofloxacin (MIC ≥16 μg/ml) was found to be associated only with the mutation of Thr-86 to Ile. The three clinical isolates UA536, UA543, and UA549 had the same mutation at Thr-86. The sequencing data also revealed that UA67 and UA549 contained third-position changes in three codons resulting in no amino acid changes (Fig. 5).

DISCUSSION

In *E. coli*, it has been demonstrated that a single mutation of Ser-83 to Ala in the GyrA protein is sufficient to render the DNA gyrase activity resistant to ciprofloxacin and nalidixic acid in vitro when the mutant protein is reconstituted with the wild-type GyrB protein (11). Using the *E. coli* paradigm, it is likely that an equivalent mutation of Thr-86 to Ile is primarily responsible for the high degree of bacterial resistance to fluoroquinolones in *C. jejuni*, although direct evidence is not yet available. It is interesting that among the four mutants of *C. jejuni* in which the critical Thr-86-to-Ile change in GyrA protein was detected, the MICs of ciprofloxacin varied from 16 to 64 μg/ml (Fig. 5). The three clinical Nal^r isolates, UA536, UA543, and UA549, whose genotypes are less well defined, appear to be more resistant to the antibiotics than the laboratory-generated mutant UA580R1. The reason for the range in antibiotic sensitivities is not clear. The susceptibility of bacteria to quinolones is certainly dependent on the permeability of the drug, the intracellular concentration of the drug, and the amino acid sequence of the target gyrase. Because we have only examined a small region of the *gyrA* gene sequence, it is possible that mutations in other locations of the *gyrA* gene, in the *gyrB* gene, or in other genes may modulate the ultimate MIC level conferred by the critical amino acid change in the target protein.

It has been reported that *C. jejuni* strains are intrinsically less susceptible to quinolones than are other enteric pathogens such as *E. coli*, *Salmonella enteritidis*, *Shigella* spp., and *Vibrio* spp. (3, 37). Gootz and Martin (9) demonstrated that the inhibitory concentration of ciprofloxacin for the supercoiling activity of the *C. jejuni* gyrase isolated from a quinolone-susceptible strain was severalfold higher than that for the *E. coli* enzyme. This finding is consistent with the bacterial susceptibility results. The lower susceptibility of *C. jejuni* to fluoroquinolones is probably due to the difference in the GyrA sequence. In *C. jejuni*, the critical residue equivalent to *E. coli* Ser-83 is a Thr-86 residue in which a methyl group replaces the hydrogen atom present in serine. A Thr residue was also found at this position in the *K. pneumoniae gyrA* gene (4), and *K. pneumoniae*, like *C. jejuni*, is also less susceptible to quinolones (37). The *K. pneumoniae gyrA* gene shares 89% overall identity with the *E. coli* gene, and within the quinolone target region examined in this study, only three other similar amino acid changes are found in addition to the Ser-Thr difference. These observations support the notion that the change in Ser-83 (*E. coli* coordinates) to Thr is critical for the one order of magnitude decrease in drug susceptibility.

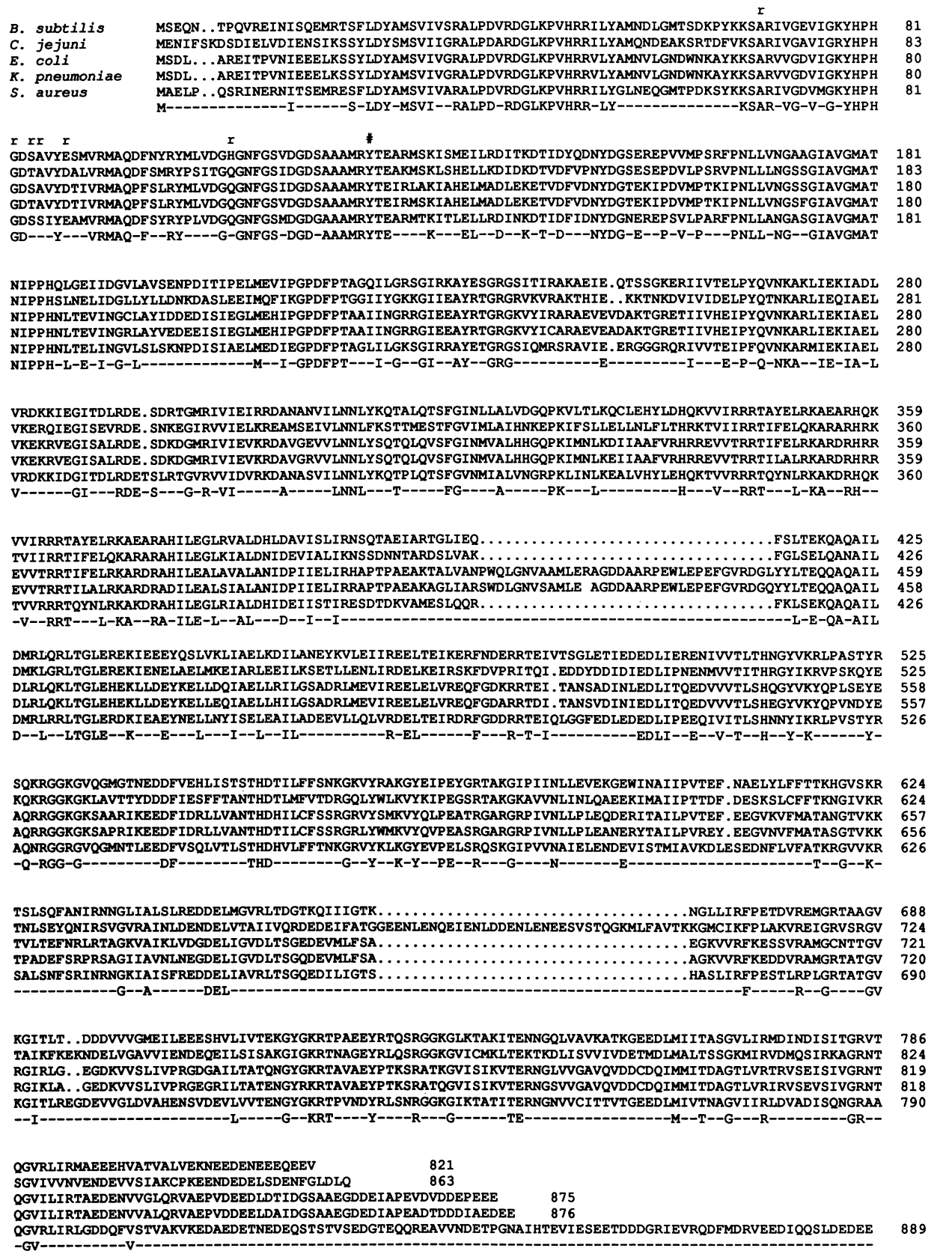

FIG. 3. Comparison of the amino acid sequences of five GyrA subunits. Data are taken from *B. subtilis* (20), *C. jejuni* (Fig. 2), *E. coli* (29), *K. pneumoniae* (4), and *S. aureus* (18). Identical amino acids in all five GyrA polypeptides are listed. Designations: r, the site in which amino acid change confers quinolone resistance; #, the active site which links to DNA.

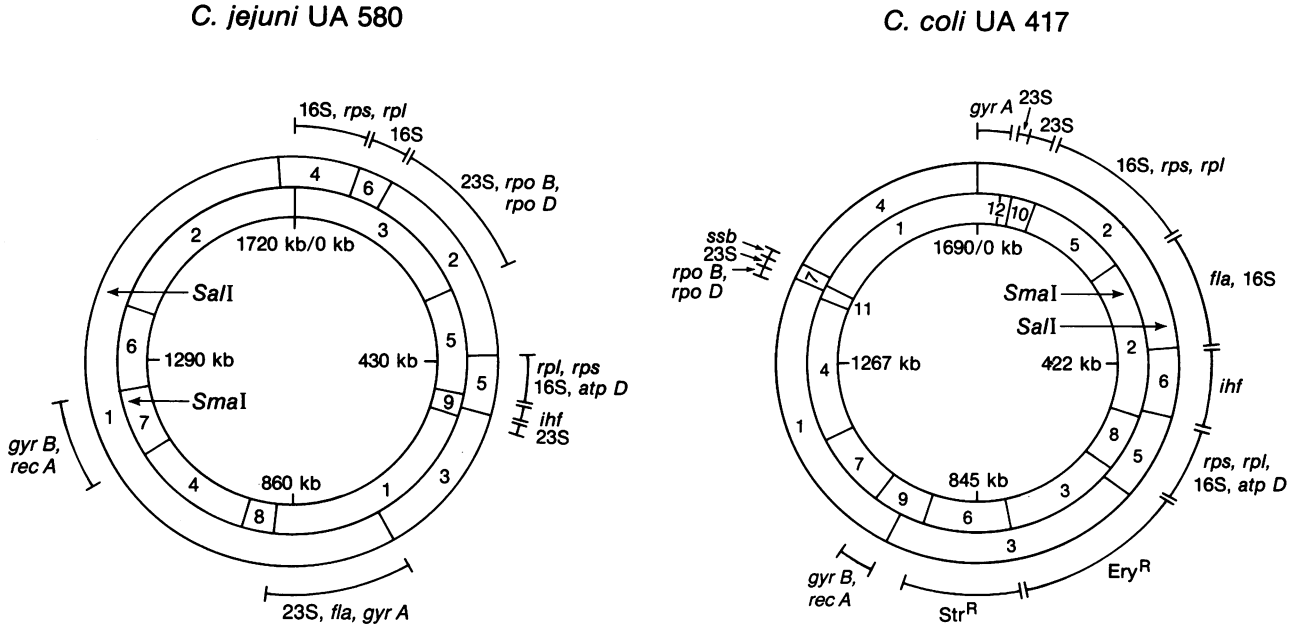

FIG. 4. Physical maps of the *C. jejuni* UA580 chromosome and the *C. coli* UA417 chromosome. Both maps are adapted from Taylor et al. (31) with the addition of the *gyrA* gene.

Recent studies have suggested that the primary binding site for quinolones is the gyrase-DNA complex, in which Ser-83 is believed to interact directly with the quinolone (19). Substitution of other amino acids for Ser-83 (or its equivalent at amino acid −86 in *C. jejuni*) would therefore be expected to lead to a reduction in binding of the quinolone to the protein-DNA complex. Our results add to the accumulating evidence that, irrespective of the detailed amino acid sequence variation in the *gyrA* gene, changes within a small region of the N-terminal domain, and most critically position Thr-86 (Ser-83 in *E. coli*), are responsible for the level of quinolone sensitivity. The knowledge of the sequence of the critical N-terminal region in the *gyrA* gene may be sufficient to provide a basis for predicting drug sensitivities in other bacteria.

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    Thr-70                                                          Ile-86
Ser Ala Arg Ile Val Gly Ala Val Ile Gly Arg Tyr His Pro His Gly Asp Thr Ala Val
TCA GCC CGT ATA GTG GGT GCT GTT ATA GGT CGT TAT CAC CCA CAT GGA GAT ACA GCA GTT
    T* (UA67)                          C (UA67)     T (UA549)          T* (UA580R1, UA536
                                                                           UA543, UA549)
    Asn-90
Tyr Asp Ala Leu Val Arg Met Ala Gln Asp Phe Ser Met Arg Tyr Pro Ser Ile Thr Gly
TAT GAT GCT TTG GTT AGA ATG GCT CAA GAT TTT TCT ATG AGA TAT CCA AGT ATT ACA GGA
    A* (UA580R3)

Gln Gly Asn Phe Gly Ser Ile Asp Gly Asp Ser Ala
CAA GGC AAC TTT GGA TCT ATA GAT GGT GAT AGT GCC
                                   C (UA67, UA549)
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Strain	Codon (Homologous to *E. coli*)	Amino acid change	MIC Nal	MIC Cip
UA580	-	Wild-Type	4	<0.5
UA580R1	86 (83)	Thr -> Ile	64	16
UA580R3	90 (87)	Asp -> Asn	32	4
UA67	70 (67)	Ala -> Thr	64	1
UA536	86 (83)	Thr -> Ile	128	32
UA543	86 (83)	Thr -> Ile	128	32
UA549	86 (83)	Thr -> Ile	64	64

FIG. 5. Mutations in *C. jejuni gyrA* genes and quinolone resistance properties. Asterisks indicate nucleotide changes that result in amino acid changes. Wobble base changes are also indicated. The strain numbers of the *C. jejuni* mutants are shown in parentheses. Nal, nalidixic acid; Cip, ciprofloxacin.

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